NANODIAMONDS ENCAPSULATED IN NANOFIBERS AS A NEW STRATEGY FOR MICRORNA-BASED THERAPY OF PROSTATE CANCER

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Abstract

Given the miRNA-34 family role as tumor suppressor in cancer, it was showed that using miRNA-34a as a new anticancer agent has a great therapeutic potential. Unfortunately, biopharmaceutical issues, rapid degradation in biological fluids, poor uptake into cells and not specific distribution into the body, prevent the use of wild-type non-coding RNA by systemic administration. To overcome these limits, in our previous study we developed a new therapeutic concept based on nanotechnology delivery of miRNA-34a into prostate cancer using fluorescent nanodiamond particles coated with polyethylenimine. In the current study, in order to achieve a more conservative, stable and controlled release of miRNA-34a, we designed a system based on nanodimaonds-miRNA34a complexes encapsulated into nanofibers. We successfully encapsulate nanodiamonds into fibers of polycaprolactione, a biodegradable polymer with a slow degradation rate and a high cellular adhesion, using an electropsinning process. We cultivated prostate cancer cells (PC3 and DU145) on nanofibers scaffolds and we analyzed the presence of nanodiamonds in cells using confocal microscopy. Moreover, in these prostate cancer cell lines we observed a replacement of miRNA-34a via real-time PCR and a decreased viability testing the metabolic activity. Our preliminar results suggest a higher increased and stable mid-term replacement of oncosuppressor miRNA-34a in prostate cancer when the nanodiamonds-miRNA complexes are delivered encapsulating them in nanofibers. Therefore we provided an effective strategy for cancer therapy ready to be tested for *in vivo* experiments.

Keywords: Nanodiamonds, nanofibers, gene therapy, prostate cancer

1. INTRODUCTION

1.1. The microRNA-34a

MicroRNAs are a class of short, single non-coding RNAs (19-22 nt), that have been identified in many eukaryotic organisms. They can prevent protein expression through cleavage of specific target mRNAs or through inhibition of their translation and may conceivably play a key role in tumorigenesis [1]. Some of miRNA molecules are associated with tumor proliferation and their level is increased inside cancer cells in comparison to healthy cells. Those miRNAs, which are contribute to carcinogenesis by inhibiting tumor suppressor genes, are considered oncogenic miRNAs (OncomiRs), while downregulated miRNAs, that normally prevent cancer development by inhibiting the expression of proto-oncogenes, are known as tumor suppressor miRNAs [2]. We focused on miRNA-34a, for which was proved and decreased level in samples from patients with prostate cancers [3]. MiR-34a may exert its tumor suppressor role via targeting several signaling molecules involved in various stages of prostate cancer progression. DNA damage and oncogenic stress strongly induced miR-34 expression in a p53-dependent pathway. The distinctive features of p53 activity are promoted by miR-34

activation: induction of cell cycle block and apoptosis through down-regulation of different proteins [4]. Given the miR-34 family role as tumor suppressor in cancer, we tried to increase the levels of miR-34a in prostate cancer cell lines through replacement with miRNA mimics transfected by *in vitro* experiments using nanodiamonds as a delivery system.

1.2. Nanodiamonds and nanofibers

The possible use of nanoparticles for diagnostic and therapeutic applications has always been very attractive. The increased interest about their usage with the increased presence of nanomaterials in commercial product has raised concerns about its potential environmental pollution and toxicity effects [5]. Nanodiamonds (ND) are carbon based nanostructures with a nanoscale size (tens to hunderds of nanometers). Given the small size of the individual nanodiamond crystals, the ratio of their surface to their volume si much greater than within the large diamonds. That means that they can be coated with large amount of functional chemicals. Their size is also suitable for transportation of molecules into the the cytoplasm of the cell. Moreover, biocompatibilty of nanodiamonds was studied under various conditions and so far nanodiamonds are considered to be non-toxic and highly biocompatible, even though no rigorous clinical trials have been done yet and the questions of expulsion of nanodiamonds or their possible accumulayion in organs remain to be answered. The use of so-called high-pressure high-temperature (HPHT) nanodiamonds has opened a possibility to track the transfection directly using engineered fluorescent nitrogen-vacancy (NV) centers. NV centers in nanodiamonds are widely used for fluorescence-based detection techniques because they provide photostability, emission in the near-infrared region and lack of photoblinking [6]. These properties have been utilized in a vast range of applications, from physics tyo biological applications such as single particle tracking inside cells or long-term *in vivo* particle tracking [7]. Finally, the HPHT fluoscencent nanodiamonds surface is negatively charged upon oxidation, and strongly binds the prototypical transfection reagent PEI for delivery of macromolecules as miRNAs. transfection reagent polyethylenimine (PEI) for delivery of biomolecules as miRNAs. Nanofibers (NF) formed by electrospinning have been shown to mimic the structure and biological function of the extracellular matrix (ECM) in terms of chemical and physical structure [8]. The diameter of electrospun fibers closely matches the size scale of the ECM, which is ideal for cell attachment, proliferation, and differentiation [9]. With respect to the stereological properties of nanofibers, fiber diameter, porosity, and pore size are the most important parameters. In addition to serving as scaffolds, nanofibers meshes can be designed to serve as delivery vehicles for bioactive factors. The main aspects for the development of successful delivery systems for tissue engineering are the bioactivity of the biomolecules incorporated within the scaffolds and the controlled release of these biomolecules according to the time frame of tissue regeneration [10]. Functionalized nanofibers have been produced directly by electrospinning polymer blends/mixtures with various chemical agents such as antibiotics, cytostatics, proteins, DNA, and small interfering RNA. Moreover, the combination of nanoparticles such liposomes with polymeric scaffolds could overcome the limitations of conventional liposomes and extend their use in drug delivery [11].

2. METHODS

PC3 and DU145 prostate cancer cell lines were maintained in RPMI1640 and DMEM media respectively supplemented with 10 % of fetal bovine serum and 1 % of antibiotics. MicroRNA-34a mimic and a scrambled microRNA (scr) as a negative control were delivered into the cells using NDs-PEI and RNAiMAx lipofectamine as a standard trasfection reagent (Tr.re.) in order to compere two different types of transfection methods. Prior to use, the nanodiamonds were dissolved in water and sonicated with a probe for 30 min. The resulting transparent colloid was filtered using a 0.2 μm PVDF microfilter. Nanodiamonds diluted in water were coated with 0.09 mg/mL of polyethylenimine (PEI 800) adding the same volume of both solutions. The mix was let vortex overnight and after the solution was centrifugated in 9000 g for 1 h to create a ND-PEI pellet. This pellet was diluted in RNAse/DNAse free water to reach a final concentration of 1 mg/μL. To link microRNA we mixed

12.5 µL of 1 mg/µL of ND-PEI with 5 µL of miRNA-34a and Scramble miRNA. Fibrous meshes were prepared using a centrifugal spinning device (Cyclone 1000 L/M Forcespinning® device; FibeRio, McAllen, TX, USA). Polycaprolactione (PCL) was dissolved in a mixture of chloroform and ethanol in a volume ratio of 9:1 to make 40 % solution, then it was miced with the nanodiamonds solution. An orifice G30 at a rotation speed of 6,000× g was used to prepare the fibrous meshes. Fibers were deposited on spunbond textile using vacuum-assisted deposition. During the electrospinning process we kept an environmental condition with 60 % of humidity. Moreover the voltage value of the intensity of the electric field between the electrode supplied by the polymer solution and the collector electrode was in range between 60 kV to 70 kV with a 60 % of humidity. Before cell seeding, PCL nanofibers were cut into round patches of 6 or 10 mm diameter and sterilized using ethylene oxide. The PC-3 and Du-145 cells were seeded for 48 and 72 h in a 96-well plate for confocal microscope visualization. Cell nuclei were stained with Hoechst 33342 and for visualization we used as excitation/emission parameters 405nm/461 (Hoechst) and 559/655-755 (NDs). Subsequently the images were recorded with an Olympus FV1000 SIM confocal microscope (objective 40×/0.95) and analyzed with Olympus FLUOVIEW 2.0a software. The cells were seeded in 96-well plates in order to evaluate cells proliferation using WST-1 assay after 48 h, 72 h and 7 days of incubation. Cells were seeded in 12-well plates and after 48 h, 72 h and 7 days incubation from the treatment total RNA was isolated. Those RNA samples were used to perform Real-time PCR in order to evaluate the expressions levels or miRNA-34 and RNU44 as a control housekeeping gene.

3. RESULTS AND DISCUSSION

3.1. Nanodiamonds-nanofibers visualization

Since fluorescent nanodiamond carriers are intended for monitoring, we performed confocal microscopy to see if it is possible to detect nanodiamonds in stained dead cells and if we can see any changes in cells seeded on nanofibers with nanodiamonds coated with miRNA-34a encapsulated. For visualization we used as excitation/emission parameters 405nm/461 (Hoechst) and 559/655-755 (ND) spectrum and any nonspecific fluorescence that interferes with nanodiamond luminescence was removed by bleach treatment. After 48 hours from the treatment, we were able to visualize nanodiamonds as clusters inside and outside nanofibers. Subsequently, at 72 hours from the incubation, we were able to visualize less nanodimonds and the nanofibers were showing a lower amount of fluorescence, probably due to the increased release of nanodiamonds. However, in the DU145 samples, it was difficult to find more clusters of nanodimonds due to the tendency that this cell line in growing as a layer of cells very close to each other's. Although, we noticed a decreased number of cells in the 72 hours samples in both cell lines, as a preliminary sign that the release of these nanodimonds with microRNA-34a was starting to affect the cell proliferation. (**Figure 1**).

3.2. Detection of miRNA-34a expression

After 48 h, 72 h and 7 days incubation from treatment with miRNA-34a and scramble miRNA transfected with both transfection methods and with encpasulation in nanofibers, we isolated total RNA and subsequently we performed Real-time PCR in order to evaluate gene expressions of miRNA-34a. We observed a significant increase of miRNA-34a expression levels in the samples treated with ND-PEI complexes with values higher than values from samples treated with the standard transfection reagent. However, the highest increase of levels of miRNA-34 was found in the PC3 cells seeded on nanofibers and after 48 h of incubation. In DU245 cell line, the replacement of miRNA-43 levels was also more successful after 48 h of incubation, however in this cell line we didn't find a significant increase of miRNA-34a levels comparing the incubation of nanodiamonds with the incubation in which the cells were seeded on nanofiers encpasulating nanodiamonds. The results were normalized using RNU44 as a control houskeeping gene (**Figure 2**).

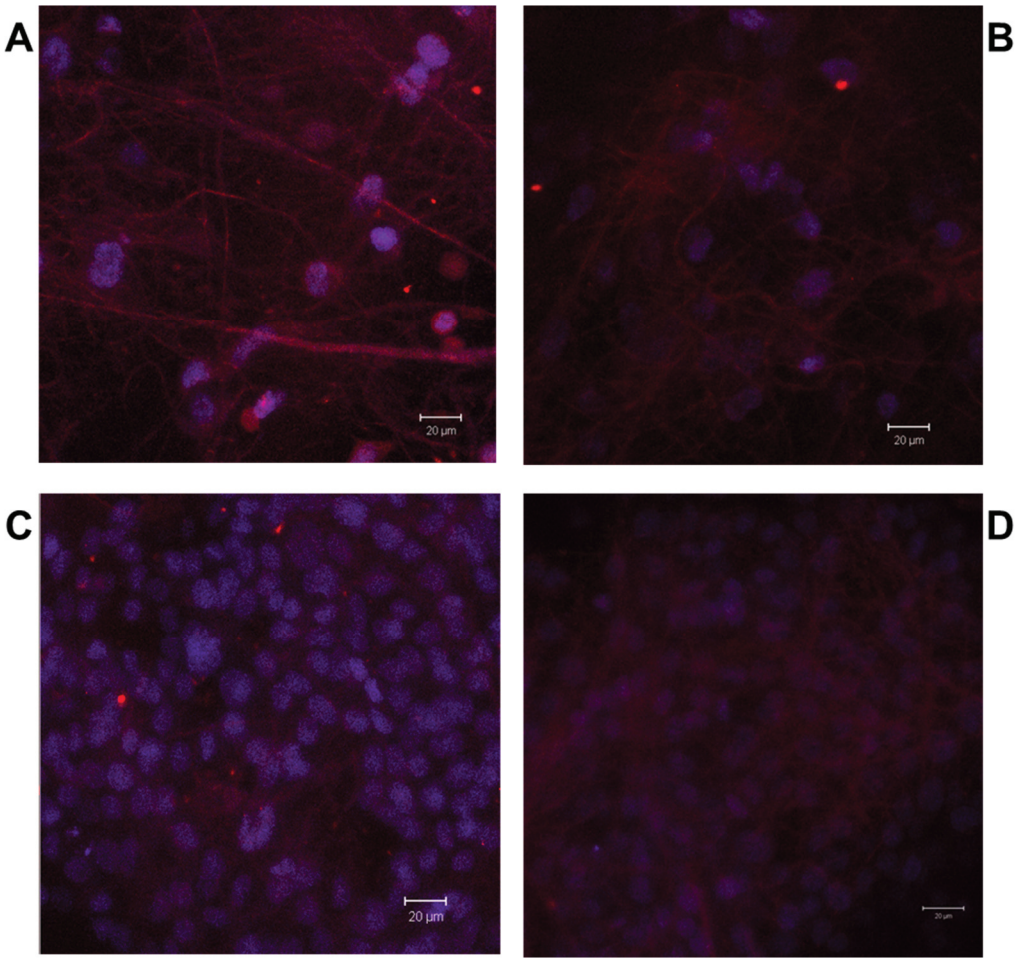

Figure 1 Images of cells seeded on nanofibers with nanodiamonds linked with microRNA encapsulated: PC3 48h (A), PC3 72h (B), DU154 48h (C), DU145 72h (D)

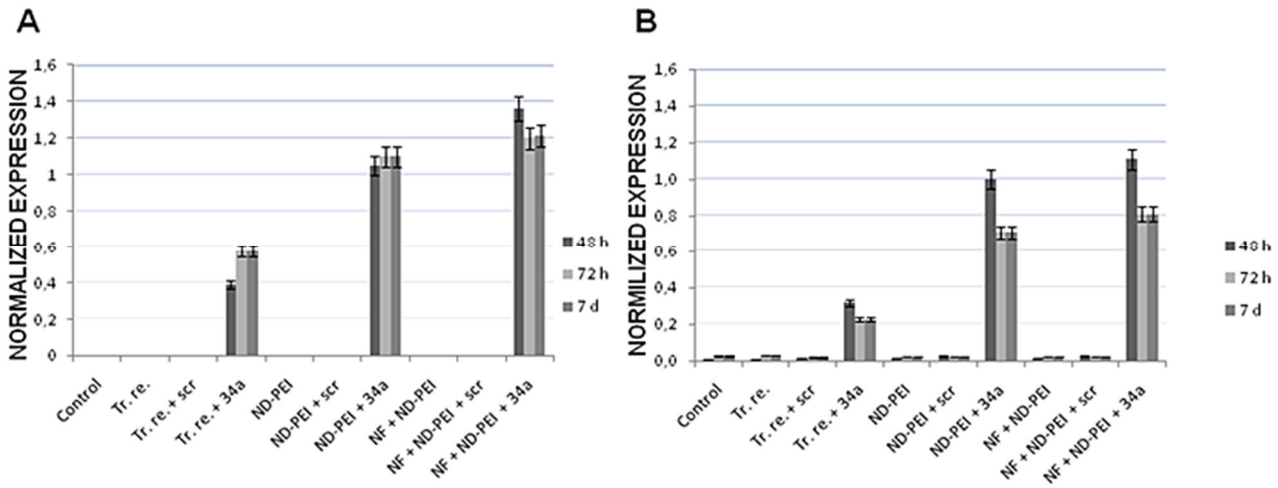

Figure 2 Real-Time PCR analysis of miRNA-34 expression in PC3 cell line (A) and DU145 cell line (B)

3.3. Evaluation of cell proliferation

The WST-1 assay was performed in order to evaluate cell proliferation in PC3 and DU145 treated with miRNA-34a and scramble miRNA transfected using ND-PEI complexes and standard transfection reagent .

Absorbance measurements were recorded after 48 and 72 h and 7 days of incubation from treatment and control samples were obteined from non-treated cells. Moreover we compared these results with the proliferation observed seeding these cell lines on nanofiber encapsulating our nanodiamonds complexes. We evaluated percentage of cell proliferation normalizing the absorbance results obteined from each samples with control samples (100 %). We observed a decrease of cell proliferation in both cell lines in samples treated with ND-PEI-miRNA34a with results similar to samples treated with the standard transfection reagent. However, the most significant difference was found when the cell lines were seeded on nanofibers after 7 days of incubation (**Figure 3**).

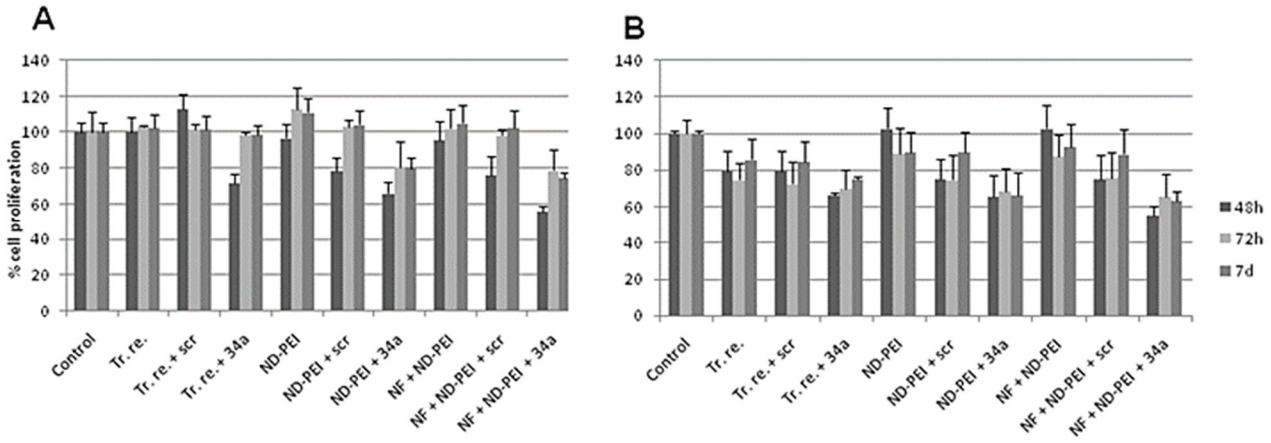

Figure 3 Cell proliferation analysis in PC3 cell line (A) and DU145 (B) cell line

4. CONCLUSION

In our previous study, we compared the transfection efficiency between a nanodiamons system and standard transfection reagent in order to delivery miRNA-34a, a well-known tumor suppressor miRNA, into prostate cancer cell lines. Our preliminary results suggested that the ND-based delivery systems seem to be a promising strategy to delivery miRNA-34a into prostate cancer in order to achieve a safe and effective targeting of the tumor. Given these promising results, in this study we observed the efficiency of such delivery system, encapsulating our nanodiamonds complexes in nanofibers of polycaprolactione made by electrospinning. We performed in vitro experiments treating PC3 and DU145 prostate cancer cell lines, and the first step was being able to visualize the fluorescence of these nanodiamonds inside the nanofibers. We noticed a decrease of fluorescence after 72 h, indicating the nanofibers' capability of releasing ND complexes coated with miRNA-34 mimic. Moreover, using WST-1 assay, we observed a significant decrease in cell proliferation in samples treated with ND-PEI-miRNA34a, especially when the cells were incubated seeding them on nanofibers and after 7 days after the treatment. However using Real-time PCR, we observed in both cell lines a very significant increase of miRNA-34a levels in samples treated with ND-PEI-miRNA34 encapsulated in nanofibers starting from the 48 h incubation time. These new results prove that seeding the cells on nanofibers encapsulating nanodiamonds can induce a constant release of these complexes even after 7 days. Overall, polycaprolactione nanofibers could be considered as a valid delivery system for a stable mid-term replacement of oncosuppressor miRNA-34a linked on fluorescent nanodiamonds.

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